Tracing and growth inhibition of Staphylococcus aureus in barbecue cheese production after product recall

Johler, Sophia ; Zurfluh, Katrin ; Stephan, Roger

Abstract: Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. It is caused by ingestion of enterotoxins formed by Staphylococcus aureus during growth in the food matrix. Following a recall of barbecue cheese due to the detection of staphylococcal enterotoxins in Switzerland in July 2015, we analyzed the production process of the respective dairy. Although most cheese-making processes involve acidification to inhibit the growth of pathogenic bacteria, barbecue cheese has to maintain a pH >6.0 to prevent undesired melting of the cheese. In addition, the dairy decided to retain the traditional manual production process of the barbecue cheese. In this study, therefore, we aimed to (1) trace Staph. aureus along the barbecue cheese production process, and (2) develop a sustainable strategy to inhibit growth of Staph. aureus and decrease the risk of staphylococcal food poisoning without changing the traditional production process. To this end, we traced Staph. aureus in a step-wise blinded process analysis on 4 different production days using spa (Staphylococcus protein A gene) typing, DNA microarray profiling, and pulsed-field gel electrophoresis analysis. We subsequently selected a new starter culture and used a model cheese production including a challenge test assay to assess its antagonistic effect on Staph. aureus growth, as well as its sensory and technological implications. We detected Staph. aureus in 30% (37/124) of the collected samples taken from the barbecue cheese production at the dairy. This included detection of Staph. aureus in the final product on all 4 production days, either after enrichment or using quantitative detection. We traced 2 enterotoxigenic Staph. aureus strains (t073/CC45 and t282/CC45) colonizing the nasal cavity and the forearms of the cheesemakers to the final product. In the challenge test assay, we were able to show that the new starter culture inhibited growth of Staph. aureus while meeting the sensory and technological requirements of barbecue cheese production.

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INTERPRETIVE SUMMARY

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Johler

Staph. aureus is one of the most prevalent causes of foodborne intoxication worldwide. Following a product recall, we investigated the barbecue cheese production process of a dairy. We traced two enterotoxigenic strains of *Staph. aureus* along the production process from colonized cheesemakers to the final product. As the dairy decided to retain the traditional production process, the most promising strategy to increase consumer safety was the implementation of a new starter culture. We were able to show that the new starter culture inhibits growth of *Staph. aureus*, while meeting the sensory and technological requirements of barbecue cheese production.

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S. Johler* , K. Zurfluh*, R. Stephan*[1]

*Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, 8057 Zurich, Switzerland

[1]Corresponding author: Roger Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland, Phone: +41-446358651, Fax: +41-446358908, E-mail: roger.stephan@uzh.ch

ABSTRACT

Staphylococcal Food Poisoning is one of the most prevalent causes of foodborne intoxications worldwide. It is caused by ingestion of enterotoxins preformed by *Staphylococcus (Staph.) aureus* during growth in the food matrix. Following a recall of barbecue cheese due to the detection of staphylococcal enterotoxins in Switzerland in July 2015, we analyzed the production process of the respective dairy. While most cheese-making processes involve acidification to inhibit the growth of pathogenic bacteria, barbecue cheese has to maintain a pH > 6.0 to avoid undesired melting of the cheese. In addition, the dairy decided to retain the traditional manual production process of the barbecue cheese. In this study, we therefore aimed to i) trace *Staph. aureus* along the barbecue cheese production process, and ii) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of staphylococcal food poisoning without changing the traditional production process. To this end, we traced *Staph. aureus* in a step-wise blinded process analysis at four different production days using *spa* typing, DNA microarray profiling, and pulsed field gel electrophoresis analysis. We subsequently selected a new starter culture and used a model cheese production including a challenge test assay to assess its antagonistic effect on *Staph. aureus* growth, as well as its sensory and technological implications. We detected *Staph. aureus* in 30% (37/124) of the collected samples taken from the barbecue cheese production at the dairy. This included detection of *Staph. aureus* in the final product on all four production days, either after enrichment or using quantitative detection. We traced two enterotoxigenic *Staph. aureus* strains (t073/CC45 and t282/CC45) colonizing the nasal cavity and the forearms of the cheesemakers to the final product. In the challenge test assay, we were able to show that the new starter culture inhibits growth of *Staph. aureus*, while meeting the sensory and technological requirements of barbecue cheese production.

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65 Key words: *Staphylococcus aureus*, contamination routes, process analysis**,** barbecue cheese,

66 starter culture

INTRODUCTION

In July 2015, the Swiss Federal Food Safety and Veterinary Office issued a warning concerning the consumption of barbecue cheese, in which *Staphylococcus (Staph.) aureus* and staphylococcal enterotoxins (SEs) had been detected. The dairy producing the barbecue cheese issued a recall, followed by a comprehensive external process analysis to trace *Staph. aureus* in the cheese-making process.

Staph. aureus can cause staphylococcal food poisoning (SFP), the most prevalent food-borne intoxication worldwide. Ingestion of major or newly-described staphylococcal enterotoxins (Hennekinne et al., 2010; Johler et al., 2015) preformed during growth of the organism in food leads to symptoms of acute gastroenteritis and violent emesis (Hu and Nakane, 2014). While symptoms usually subside within 24 hours, SFP can in rare cases be fatal for children and the elderly. The CDC estimates 240,000 cases per year in the US, resulting in 1,000 hospitalizations and six deaths (Scallan et al., 2011).

As SEs are heat stable and will not be inactivated during the cooking process, preventive measures focus on inhibiting growth of *Staph. aureus* in the food matrix (Le Loir et al., 2003). To this end, starter cultures are used in the production of a wide range of foods including cheese. A suitable starter culture will outcompete the organism, thus effectively preventing *Staph. aureus* growth and SE formation. However, starter cultures need to meet several criteria to be suitable for the production of barbecue cheese. While the antagonistic effect of many starter cultures is due to acidification of the food matrix, the pH of barbecue cheese cannot be lowered to values < 6.0, as this would result in melting of the cheese when it is exposed to high temperatures during preparation by the customer. In addition, suitable starter cultures must have no negative sensory implications.

In this study, we aimed to i) trace *Staph. aureus* in the barbecue cheese production process, and ii) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of SFP without changing the traditional production process.

MATERIALS AND METHODS

Sampling along the production process and isolation of coagulase-positive staphylococci

To identify potential sources for contamination of the barbecue cheese at the dairy, four production cycles (see Figure 1) of the barbecue cheese were screened for coagulase-positive staphylococci in a blinded setup. To avoid bias, the dairy and the cheesemakers were only informed of the results upon completion of the study. Samples were taken from cheesemakers and at each step of the cheese-making process on Aug. 24th (T_1), Aug. 27th (T_2), Sept. 1st (T_3), and Sept. 3rd (T_4), 2015 (see Table 1). Before the start of the cheese production, swabs from the anterior nares and forearms of the cheesemakers were taken, as well as a swab from the inner and outer side of the end of the milk hose after pasteurization. All swabs were moistened using 0.85% NaCl. During the cheese production, 10 mL samples of milk, whey, and starter cultures, and 30 g samples of curd and cheese were taken. Samples were screened for coagulase positive staphylococci (CPS) using enrichment in Mueller-Hinton broth with 6.5% NaCl and plating on rabbit plasma fibrinogen (RPF) agar (Oxoid, Pratteln, Switzerland), and quantitatively following the EN ISO 6888-2 protocol. *Staph. aureus* species identification was achieved through subsequent *spa* typing and the detection of species-specific markers by Staphytype DNA microarray profiling.

Cell Lysis and DNA Extraction

For cell lysis and DNA extraction, reagents of the Staphytype genotyping kit 2.0 (Alere, Jena, Germany) and the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) were used according to the manufacturers' instructions. The concentration of nucleic acids was measured using a Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Spa Typing

The polymorphic X region of *spa* was amplified as previously described (Wattinger et al., 2012). Each PCR product was subsequently purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Buchs, Switzerland) and sequencing was outsourced (Microsynth, Balgach, Switzerland). Subsequently, *spa* types were determined using the *spa*-server (http://spa.ridom.de/) (Harmsen et al., 2003).

DNA Microarray Based Genotyping

The Staphytype genotyping kit 2.0 was used to detect the presence/absence of over 300 virulence and resistance genes and their allelic variants in *Staph. aureus* strains that were traced from the cheesemakers to the final product. Detection included genes encoding the major staphylococcal enterotoxins (*sea, seb, sec, sed, see*), as well as genes coding for newly described staphylococcal enterotoxins and enterotoxin-like superantigens (*seg, seh, sei, sej, sek, sel, selm, seln, selo, seq, ser, selu*). Microarray profiles also allowed for assignment of the strains to *agr* types, as well as clonal complexes (CC) (Monecke et al., 2008).

Pulsed-field gel electrophoresis (PFGE) analysis

138 Preparation of chromosomal DNA and PFGE analysis of *Sma*I digested fragments was
139 performed as previously described (Bannerman et al., 1995). Electrophoresis was carried out in a
140 Bio-Rad CHEF-DR III electrophoresis cell. *Salmonella enterica* serovar Braenderup strain
141 H9812 digested with 50 U *Xba*I (12 h, 37°C) was used as a molecular size standard. Gels were
142 analyzed with Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) using the
143 dice coefficient and were represented by unweighted pair grouping by mathematical averaging
144 (UPGMA) with an optimization of 0.5%, and position tolerance of 1%.
145
146 ***Model Cheese Production Including Challenge Test Assay Using New Starter Culture***
147 A new starter culture, consisting of coagulase-negative staphlyococci (CNS), was chosen
148 (START Crudo 500, Christl Gewürze GmbH, Moosdorf, Austria). CNS species identification
149 was performed by matrix-assisted laser desorption-ionization – time of flight mass spectrometry
150 (MALDI-TOF MS). To evaluate the suitability of the new starter culture for the barbecue cheese
151 production, the cheesemakers produced four batches of model cheese (batches A-D) in a
152 laboratory setting.
153 To assess growth of the starter culture during the cheese-making process, we determined CNS
154 counts in two barbecue cheese production batches (batches A and B) at three time points. The
155 first sample was taken from the curd directly after coagulation, the second sample was obtained
156 from the cheese after pressing (same day), and the third sample was taken in the morning of the
157 following day. At this time, we also measured the pH of the cheese.
158 The inhibitory effect of the new starter culture on *Staph. aureus* growth was assessed in a
159 challenge assay using two batches of barbecue cheese (batches C and D) produced with milk
160 contaminated with *Staph. aureus*. To this end, a *Staph. aureus* strain isolated during the step-wise
161 analysis of the barbecue cheese production process (strain 1, P30, t282) was grown in BHI broth

at 37°C (225 rpm shaking) over night to 2.5 x 10^9 CFU/mL. From the overnight broth, as well as from its 10-fold dilution in 0.85% NaCl, 1.0 mL were added to 3 L of milk, resulting in contamination levels of 10^6 CFU/mL milk in batch C and 10^5 CFU/mL milk in batch D. The cheeses were sampled after 24 h and CPS counts were determined following the EN ISO 6888-2 protocol.

RESULTS AND DISCUSSION

We detected *Staph. aureus* in 30% (37/124) of the collected samples which were taken along the production process of the four barbecue cheese production cycles at the dairy (see Table 1). This included detection of *Staph. aureus* in the final product on all four production days, either after enrichment (T_1) or by quantitative detection methods (T_2-T_4). The highest CPS count detected in a sample equaled 6.3 x 10^5 CfU/g in the final product, with cell density levels of 10^5 to 10^6 CFU/mL generally being regarded as sufficient for enterotoxin production. The step-wise analysis showed that in most cases low-level contaminations occurred early in the production process, with *Staph. aureus* being detected only after enrichment.

To determine the source of the contamination, we traced the isolates along the barbecue cheese production process using *spa* typing, DNA microarray analysis, and PFGE analysis. The 37 isolates represented six different *spa* types: t282 (n = 15), t073 (n = 15), t5264 (n = 4), t362 (n = 1), t529 (n = 1), and t3992 (n = 1). The three *spa* types that were detected only once were found in *Staph. aureus* strains obtained from raw milk and the forearm of a cheesemaker. As these strains were not present in the final product, we excluded them from further characterization experiments. DNA microarray analysis and *spa* typing showed that all remaining 34 isolates could be assigned to three distinct *Staph. aureus* strains. Genotypic characteristics, including typing results, as well as enterotoxin gene profiles of these strains are provided in Table 2. Strains

1 (t282/CC45) and 2 (t073/CC45) harbored enterotoxin genes and were repeatedly detected in samples from cheesemakers and in the final product. Strain identity of selected isolates was confirmed by PFGE analysis, resulting in only two distinct patterns. A third *Staph. aureus* strain (t5264/CC88) was only found in one production cycle (T4) and was shown not to be enterotoxigenic.

Staph. aureus colonizes the skin and mucosa of humans, with nasal carriage rates between 30% and 50% in the adult population (Wattinger et al., 2012; Diederen et al., 2006; Halablab et al., 2010; Munckhof et al., 2008). The three *Staph. aureus* strains detected in the nasal cavity and on the forearms of the cheesemakers, as well as in the final product, were assigned to t282/CC45, t073/CC45, and t5264/CC88. *Staph. aureus* isolates of these *spa* types and clonal complexes have been previously reported in association with asymptomatic colonization and cases of infections in humans (Bloemendaal et al., 2009; Luedicke et al., 2010; Wattinger et al., 2012; Gómez-Sanz et al., 2013). *Staph. aureus* nasal carriage status is not necessarily permanent, but was shown to frequently change over time (Sakwinska et al., 2009). While decolonization strategies such as intranasal application of mupirocin and chlorhexidine washing have high success rates immediately after treatment, many carriers will become recolonized during a longer follow-up period (Ammerlaan et al., 2009; van Rijen et al., 2008). Thus, decolonization of cheesemakers does not represent a sustainable approach to increase food safety and promotes the risk of nasal colonization with mupirocin-resistant *Staph. aureus* (Ammerlaan et al., 2009).

As the dairy decided to retain the traditional manual production of the barbecue cheese, the most promising strategy of increasing food safety and reducing the risk of SFP was the inhibition of *Staph. aureus* growth by use of a new starter culture. The candidate starter culture was identified as *Staph. vitulinus* by MALDI-TOF MS. As shown in Table 3, the new starter culture was able to grow under the conditions of the cheese-making process. It had no negative sensory implications

and resulted in a favorable pH of the product (pH = 6.29). In the challenge assay, we also observed that the starter culture was able to outcompete *Staph. aureus* (Table 4), with a maximum increase of CPS counts of 1 $\log_{10}$. This is particularly remarkable, as physical concentration effects alone can result in an increase of 1 $\log_{10}$ during the cheese-making process (Peng et al., 2013).

CONCLUSIONS

We conclude that while the pasteurization process of the raw milk used for barbecue cheese production was successful, there is still the risk of SFP due to *Staph. aureus* contamination by colonized cheesemakers. As decolonization of food handlers cannot assure long-term freedom from nasal or skin carriage, the most promising strategy to increase food safety in the traditional manual production process is the use of a suitable starter culture. We were able to show that the new *Staph. vitulinus* starter culture inhibited growth of *Staph. aureus*, while meeting the sensory and technological requirements of barbecue cheese production. The dairy has by now successfully implemented the new starter culture in the production process of the barbecue cheese and resumed regular production.

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298 **TABLES**

299 Table 1: Tracing *Staph. aureus* in a step-wise blinded process analysis at four different days (T_1-
300 T_4). The table indicates at which production steps *Staph. aureus* isolates were detected and
301 provides an overview of the respective *spa* types (t073, t282, t5264, t529, t3992) and PFGE
302 patterns ($^{A, B}$) determined for selected isolates. Three strains could be traced from the
303 cheesemakers to the final product (t072^{A}, t282^{B}, t5264).

ID	Source	Sampling details	Result[1,2]			
			T_1	T_2	T_3	T_4
Before processing (swabs)						
BP1	Cheesemaker 1	Nose	t282^{B}	-	t282^{B}	t282^{B}
BP2	Cheesemaker 2	Nose	t073^{A}	t073^{A}	t073^{A}	t073^{A}
BP3	Cheesemaker 1	Forearms	t282	t362$_{e}$	-	-
BP4	Cheesemaker 2	Forearms	-	t073^{A}	-	t5264
BP5	Milk hose after pasteurization	Inside and outside	-	-	-	-
Processing						
P1	Raw milk		t529	t3992	-	-
P2	Milk after pasteurization	Production start	-	-	-	-
P3	Milk after pasteurization	Mid-production	-	-	-	-
P4	Starter culture		-	-	-	-
P5	Curd before cutting	Sample 1	-	-	-	-
P6	Curd before cutting	Sample 2	-	-	-	-
P7	Curd before cutting	Sample 3	-	-	t073$^{A}_{e}$	-
P8	Curd after 1st stirring	Sample 1	-	t073$^{A}_{q}$	-	t073$^{A}_{e}$

P9	Curd after 1st stirring	Sample 2	-	-	-	-
P10	Curd after 1st stirring	Sample 3	-	$t073_e$	-	-
P11	Whey	1st draining	-	-	-	-
P12	Curd after 2nd stirring	Sample 1	-	-	-	$t282^B_e$
P13	Curd after 2nd stirring	Sample 2	-	-	-	$t073^A_e$
P14	Curd after 2nd stirring	Sample 3	-	-	-	-
P15	Whey	2nd draining	-	-	-	-
P16	Curd after mixing	Sample 1	-	-	-	-
P17	Curd after mixing	Sample 2	-	-	-	-
P18	Curd after mixing	Sample 3	-	-	-	-
P19	Whey	3rd draining	-	-	-	$t282^B_e$
P20	Cheese after pressing I	Sample 1	-	-	-	-
P21	Cheese after pressing I	Sample 2	-	-	-	$t282_e$
P22	Cheese after pressing I	Sample 3	-	-	-	$t282_e$
P23	Cheese after pressing II	Sample 1	-	-	-	$t5264_q$
P24	Cheese after pressing II	Sample 2	-	-	-	$t282_e$
P25	Cheese after pressing II	Sample 3	-	-	-	$t073_e$
P26	Cheese after pressing III	Sample 1	-	-	-	$t282_q$
P27	Cheese after pressing III	Sample 2	-	-	-	$t5264_q$
P28	Cheese after pressing III	Sample 3	-	-	-	$t282_q$
P29	Cheese after pressing IV	Sample 1	-	$t073^A_q$	-	$t5264_q$
P30	Cheese after pressing IV	Sample 2	-	$t073_q$	$t282^B_q$	$t282_q$
P31	Cheese after pressing IV	Sample 3	$t282_e$	$t073_q$	$t073^A_q$	$t282_q$

[1] Superscript letters indicate assignment of isolates to PFGE patterns

[2] Quantitative detection ($_q$) is possible for CPS counts > 10^2 CFU/g. Lower levels are only detectable after enrichmant ($_e$).

Table 2: Molecular characteristics of the three *Staph. aureus* strains traced from the cheesemakers to the final product.

		Strain 1	Strain 2	Strain 3
Typing	*spa* typing	t282	t073	t5264
	PFGE	Pattern B	Pattern A	not determined
	Clonal complex	CC45	CC45	CC88
	agr typing	*agr*I	*agr*I	*agr*III
Enterotoxin genes	Major enterotoxins[2]	-	*sec*	-
	Others[2]	*seg, sei, selm, seln, selo, selu*	*seg, sei, sel, selm, seln, selo, selu*	-

[1] Screening included all major enterotoxin genes (*sea, seb, sec, sed,* and *see*).

[2] Screening included *seg, seh, sei, sej, sek, sel, selm, seln, selo, seq, ser,* and *selu*.

Table 3: Growth of the starter culture was evaluated by CNS counts using a model cheese production. Growth in presence of *Staph. aureus* was determined using an initial milk contamination level of 10^3 CFU *Staph. aureus* per mL milk.

Time point	Batch A		Batch B (+ *Staph. aureus*)
	CNS[1]	pH	CNS[1]
After coagulation	1.4 x 10^6	-	1.3 x 10^6
After pressing	2.2 x 10^5	-	2.5 x 10^5
Next morning	2.9 x 10^7	6.29	6.0 x 10^7

[1] Counts indicated as CFU/ g

317

318 Table 4: Challenge test assay assessing inhibition of *Staph. aureus* growth by the new starter
319 culture in a model cheese production. Growth of *Staph. aureus* and the new starter culture was
320 quantified using CPS and CNS counts, respectively.

	CPS		CNS
	artificial contamination in milk	after 24h	after 24 h
Batch C	10^6 CFU/mL	2.0 x 10^7 CFU/g	2.4 x 10^8 CFU/g
Batch D	10^5 CFU/mL	6.0 x 10^5 CFU/g	2.1 x 10^8 CFU/g

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325 **FIGURES**

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327 Figure 1: Flow chart providing an overview of the barbecue cheese production process until the
328 ripening stage. The average batch size equals 500 kg of milk resulting in 55 kg of cheese.

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